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1. PROC. INSTRUMENT IDEN. (CONTRACT)	(ORDER) NO	6. INVOICE NO. / DATE	7. PAGE	OF	8. ACCEPTANCE POINT
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MICROBIAL INSIGHTS INC. 2340 STOCK CREEK BLVD ROCKFORD, TN 37853-3044	Ø7MYØ	DCMAO ATLANTA 805 WALKER STREET MARIETTA, GA 300060-2789	S1103A

11. SHIPPED FROM (If other than 9)	CODE	FOB	12. PAYMENT WILL BE MADE BY	CODE
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PROGRAM OFFICER (ATTN: KEITH WARD) OFFICE OF NAVAL RESEARCH 800 NORTH QUINCY STREET ARLINGTON, VA 22217-5660	ONR 331	PROGRAM OFFICER (ATTN: KEITH WARD) OFFICE OF NAVAL RESEARCH 800 NORTH QUINCY STREET ARLINGTON, VA 22217-5660	ONR 331

15. ITEM NO	16. STOCK/PART NO. DESCRIPTION (Indicate number of shipping containers - type of container - container number)	17. QUANTITY SHIP / REC'D *	18. UNIT	19. UNIT PRICE	20. AMOUNT
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Application of molecular techniques to define mechanisms of microbially influenced corrosion of stainless steel and copper in marine systems (N 00014-95-C-0299)

James A. Coyer, David C. White

Final Report

August 1997

Bacteria play a major role in the processes of biofouling and biocorrosion as members of biofilms and regulators of nutrient cycling in these systems. In these capacities, bacterial communities change temporally and spatially due to consortial interactions. Some bacterial species may facilitate or inhibit development of metabolic capacity of other bacterial species in the biofilm. To fully understand the processes of consortial interactions, therefore, the identity and activity of component species in a consortium must be resolved at the relevant temporal and spatial scales.

The objectives of our research were to resolve the temporal and spatial patterns of N_2-fixing and sulfate-reducing bacteria within a bacterial consortium. Specifically,

1. Develop *in situ* hybridization techniques (*in situ* PCR) with fluorescent probes to detect the temporal abundance and spatial distribution of N_2-fixing, and sulfate-reducing bacteria in biofilms

2. Map the spatial distribution of the bacteria using congruently *in situ* hybridization techniques and confocal microscopy

Several approaches were directed toward Objective 1. First, a biofilm was simulated by smearing fixed bacterial cells onto a glass microscope slide. The first experiment used immuno *in situ* techniques, whereby the anti-nitrogenase antibody was applied to a slide smeared with: a) *V. natriegens* cells that were harvested when fixing N_2, b) *V. natriegens* cells that were not fixing N_2, and c) the sulfate-reducing bacterium (SRB), *D. vulgaris*. A strong fluorescent signal was present in the N_2-fixing cells, but not in the non-N_2-fixing cells or the SRB. The same assemblage of cells also was probed with EUB, a probe specific for a 18 bp conserved region of the SSU in all eubacteria. The 18 bp/rhodamine-coupled probe produced a strong signal in both *V. natriegens* and *D. vulgaris*. Thus, we are able to introduce antibody and DNA probes into whole bacterial cells fixed onto glass slides.

Secondly, we used direct *in situ* PCR techniques to detect naturally and PCR-amplified nucleic acid targets within bacterial cells fixed onto glass slides. Detection of naturally-amplified targets (rRNA) produced equivocal results as the SRB-specific probe (SRB-385; Amann et al. 1992. Appl. Environ. Micro.58:614-23) did not always produce a signal in the SRB *D. vulgaris*. Direct *in situ* PCR detection of PCR-amplified targets, however, regularly produced signals. Primers (L1, G1) were used to both amplify and label (with DIG) the spacer region between the SSU and LSU in fixed cells of *Vibrio* and the amplified target was detected by a primary antibody (anti-DIG) and a colorimetric assay.

Third, we used direct and indirect PCR techniques to detect low-copy number targets (protein-coding genes) within bacterial cells in solution or fixed onto glass slides. In the direct method, PCR primers (Hyd1, Hyd5) were used to both amplify and label (with DIG), a 1 kb region of the [NiFe] hydrogenase gene in fixed cells, both in solution and on slides, of *Desulfovibrio vulgaris*. The amplified region was detected with a primary antibody (anti-DIG) and a colorimetric assay, as well as with the primary antibody coupled to the fluorochrome, rhodamine.

In the indirect method, the primers (Hyd 2, Hyd 5) were used to amplify the 1 kb region of the hydrogenase gene which subsequently was detected with a rhodomine-labeled probe (Hyd 1). The advantage of the indirect over the direct approach to *in situ* PCR hybridization is that the former results in far fewer false positives.

We also used a two-species system consisting of N_2-fixing cells and SRB. N_2-fixing can be detected by the immuno assay described above and all fixed cells (N_2-fixing and SRB) can be detected with a DNA stain (propidium

iodide). Each type of cell can be distinguished by the different emission wavelengths from the fluor-coupled nitrogenase antibody and the propidium iodide: cells with both signals (N_2-fixing) emit a different wavelength than cells with only propidium iodide (SRB). We have successfully applied this technique to a simulated biofilm consisting of N_2 and SRB cells.

Applying immuno and direct and indirect PCR techniques to natural biofilms grown in special flowcells (Palmer and Caldwell. 1995. J. Microbiol. Methods 24:171-182), however, proved problematic. As the volume of the flowcells (250 μL) was much larger than the volume used for PCR on simulated biofilms ((25 μL), the subsequent 10-fold increase in the PCR reaction mix lead to negative results. Furthermore, investigation revealed that application of fixatives and the various reagents and washes necessary for *in situ* PCR hybridization invariably distorted or removed the biofilm. The flowcell design has been modified and the new design currently is being evaluated.

In summary, detection of specific types of bacteria within a biofilm may be best accomplished using fluorescent *in situ* hybridization (FISH) techniques employing probes of fluorochrome-coupled antibodies, rather than using either direct or indirect *in situ* PCR hybridization techniques to amplify regions of DNA. Immuno *in situ* techniques are faster, and because of the specific antibody-antigen reaction, produce very little background and/or false-positive signals . As long as antibodies are available for proteins indicative of specific physiological traits (nitrogenase for N_2-fixing, hydrogenase for sulfate-reducing) expressed by members of a biofilm consortium, clear "yes-no" answers can be formed.

In situ PCR techniques, however, frequently produce confusing results because of high levels of background and/or false-positive signals, both of which develop in spite of stringent reaction conditions. Indeed, the stringent reaction conditions may be so specific as to preclude development of a "universal" protocol for the ever-changing assemblage of species in a consortium. The expense of purifying proteins for antibody production is not trivial, but once developed, the highly specific antibody-antigen reaction will lead to a more rigorous and unequivocal approach to mapping component species in a biofilm consortium.